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Tuber petrophilum, a new truffle species from Serbia

Miroljub Milenković[1], Tine Grebenc[2],
Miroslav Marković[3] & Boris Ivančević[4*]

[1]Institute for Biological Research "Siniša Stanković"
Bulevar despota Stefana 142, RS-11060 Belgrade, Serbia
[2]Slovenian Forestry Institute, Večna pot 2, SI-1000 Ljubljana, Slovenia
[3]Banatska 34, RS-26340 Bela Crkva, Serbia
[4]Natural History Museum, Njegoševa 51, RS-11000 Belgrade, Serbia
* Correspondence to: *i.boris@beotel.net*

Abstract — *Tuber petrophilum* sp. nov., within the *Tuber melanosporum* lineage, is described from Mount Tara (western Serbia) based on morphological and ITS molecular data. It is recognizable by its minute ascomata that produce ovoid to ellipsoid to subfusiform spores bearing aculeate ornamentation. Among black truffles, the new species is distinguished by its irregularly roundish to subglobose ascomata not exceeding 1.6 cm in diameter, with a basal depression or cavity and peridium surface which appears as a thin semi-transparent layer while fresh. The species forms a monophyletic well-supported clade in Maximum Likelihood ITS phylogeny, closely related to *Tuber brumale* aggr. The distinctive feature of the new species lies in its specific and unique microhabitat, limited to humus-rich substrata accumulated as soil pockets in limestone rocks, commonly 20–100 cm above the continuous forest soil terraces. The species description is supplemented with macro- and micro-photographs, and a key to the species of the *T. melanosporum* lineage.

Key words — biodiversity, ecology of truffles, hypogeous fungi, *Tuberaceae*

Introduction

On several occasions between 2004 and 2014, the first author with associates explored hypogeous fungi in the Tara National Park on Mount Tara, which is located in western Serbia on the edge of northeastern belt of the Dinaric Alps. This area is a significant refugium for many endemic and relict plant species (Stevanović et al. 1995; Cvjetićanin & Novaković 2007, 2010). Apart from several hypogeous species found in quite usual habitats that were already known from other areas of Serbia, a few black truffles were discovered in an unusual and atypical habitat: cavities, crevices, and recesses in limestone rocks.

These specimens were readily distinguishable by a unique aroma, ascomatal size, and structure that did not correspond to any previously described species.

This paper aims to (1) describe a new species based on both morphological and molecular data, (2) ascertain the ecology of the species, and (3) provide an identification key and a phylogram to the *Tuber melanosporum* lineage.

Materials & methods

Sampling sites and collection treatment. — Our morphological examination and description of *Tuber petrophilum* is based on eleven collections comprising 26 ascomata from five localities in western Serbia on Mount Tara during September and October, 2004–14. All sites are situated within a narrow belt, a few hundred meters wide, along the local Mitrovac–Kaluđerske Bare road. The distance between the two furthest sites is approx. 6.7 km. Ascomata were discovered by a trained dog. Vouchers are conserved in the fungal reference collection of the Natural History Museum, Belgrade, Serbia (BEO), the fungal reference collection of Ljubljana University, Slovenia (LJU), and the private herbarium of M. Marković, Bela Crkva, Serbia. Ascomata were preserved by drying or by ethanol-glycerin fixative (70% ethanol to which 15% of glycerol was added). Sampling was done in representative forest communities in a limestone-dominated area where hypogeous fungal biodiversity was completely unknown. Climate data are available for the nearby meteorological station at Mitrovac (1082 m asl). Over the last 25 years, the yearly average temperature at the station was 5 °C and mean annual precipitation was 980 mm.

Description of hues uses the Pantone colour standard (Eiseman & Lawrence 1990). The letter "P" followed by the number in parentheses represents the Pantone code. Microscopic characters of *Tuber petrophilum* were examined in both fresh and dry material with a Leica Leitz DMRB microscope equipped with a Leica DFC320 digital microscope camera and Leica DMLS microscope equipped with Leica DFC295 digital microscope camera. Spores were measured in 5% KOH in a bright field microscopy using Zeiss Axio Imager.Z2 microscope (100–1000× magnification) with the automated measuring function in ZEN pro 2011 software. Spores considered to be not full-size, anomalous, or aberrant were excluded. The length and width of full-sized spores were measured in side view. Ornamentation was excluded from spore measurements and measured separately. The spore size quotient Q represents the length : width ratio range determined from all measured ascospores. The mean quotient Q_m was calculated as arithmetic average of the Q values from all ascospore measured. Twenty spores were measured for each collection.

As truffles are known for their specific soil requirements, several basic soil characteristics at the three most productive localities (Dolak [the holotype site], Tisovo brdo, and Rustine), were analyzed. About 0.5 L of soil was taken around the ascomata and analyzed for humus, phosphorus (P_2O_5) and potassium (K_2O) content, and pH. Standard laboratory analyses of collected soil samples included the Kotzman method for humus content, measurement of pH by placing a glass electrode in 1M KCL solution, Egner-Rhim Al flame photometry method for potassium, Egner-Rhim Al colorimetry method for phosphorus, and determination of calcium carbonate by

Scheibler volumetric method (Tan 1998). Analyses were performed at the Institute for Soil Science (Belgrade, Serbia).

DNA EXTRACTION, PCR AMPLIFICATION AND SEQUENCING. — DNA was extracted from four dried glebal samples from a mature and sufficiently large *Tuber petrophilum* ascoma using a Qiagen DNeasy Plant Mini Kit. DNA amplification using general ITS primers ITS1f combined with ITS4 (White et al. 1990, Gardes & Bruns 1993) followed the modified protocol in Grebenc et al. (2009). Prior to sequencing, products were cleaned from the gel using the Promega Wizard SV Genomic DNA Purification System. Both strands were sequenced separately at Macrogen Korea (Seoul, Korea). All samples were sequenced in both directions using the same primers as in the amplification. Sequencher 5.1 was used to assemble the consensus sequence from the two strands of the ITS nrDNA of each isolate. Complete ITS sequences were deposited in GenBank under accession numbers HG810883–810886.

PHYLOGENETIC ANALYSES. — All obtained consensus sequences were aligned with a selection of *Tuber* sequences from the International Nucleotide Sequence Databases using the stand-alone freeware version of the MAFFT programme (http://align.bmr.kyushu-u.ac.jp/mafft/software/) with the E-INS-i aligning strategy (Katoh et al. 2005). *Reddellomyces* sp. (GenBank: HM485336 and HM485337) was used as outgroup as a close *Tuber* relative from the southern hemisphere with a basal position according to Bonito et al. (2013). The MEGA 5.10 package was used to construct Maximum Likelihood (ML) phylogenetic reconstruction based on a Tamura-3-parameter substitution model with Gamma distributed rates among sites including invariant sites. In the dataset gaps / missing data were partially deleted and 95% site coverage cutoff and a strong branch swap filter were applied. 2000 bootstrap replicates were run. The Maximum Parsimony (MP) reconstruction and branch support was based on a MEGA 5.10 incorporated Subtree-Pruning-Regrafting MP search method with 2000 replicates, gaps / missing data were partially deleted, and a 95% site coverage cutoff was applied. MEGA 5.10 package was used also to annotate the final tree and collapse branches at species level.

Taxonomy

Tuber petrophilum Milenković, P. Jovan., Grebenc, Ivančević & Marković, sp. nov.

PLATE 1

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Differs from all other species of the *Tuber melanosporum* lineage by its considerably smaller mature ascomata, its reddish-black peridium with a thin semi-transparent layer when fresh, its spores with shorter ornamentation, and its distinctive habit in accumulated soil pockets in rocks.

TYPE: Serbia, Bajina Bašta, Tara National Park, Mitrovac, Dolak, 43.924810°N 19.462610°E, 1015 m asl, under *Fagus sylvatica* & *Abies alba*, in humus-rich substrate accumulated as soil pockets in limestone rocks, commonly 20–100 cm above the continuous forest soil terraces, 5 ascomata, 28 Sep 2004, leg. M. Milenković (**Holotype,** BEO 20600).

Etymology: in reference to the specific habitat of the species – *petrophilum* (Gr. πέτρα = stone, rock; φίλος = who love).

Ascomata strictly hypogeous, solitary or in small groups, rather small, ranging from 8–16 mm in diam., nearly globose to irregularly subglobose, with basal depression or cavity, firm.

Peridium reddish-black (P 19-1015), when fresh, surface appears as a thin semi-transparent layer, turning to non-transparent and completely black when dried, pseudoparenchymatous, total thickness 150–450 µm (average 320 µm), with melanised outer layer, 20–200 µm, without hyphal outgrowth. Warts angular, flattened and depressed at the apex, 0.6–1.2 mm diam., 0.3–0.7 mm high.

Gleba solid, whitish at first, becoming brown (P 18-1137) and finally dark brown (coffee brown; P 18-1154), at maturity with wide, infrequently (1–3×) branched white veins, which radiate from the base and terminate close to the peridium. Asci from fresh ascomata subglobose to irregular with well-developed stalk, (1–)2–4(–6, extremely rarely)-spored, 40–50 × 55–80 µm, spores when inside asci randomly disposed and loosely packed. Ascospores 22.7–42.5 × 13.1–26 µm, symmetrical to asymmetrical, ovoid to ellipsoid to subfusiform, Q = 1.37–2.29, Q_m = 1.68 (SD = 0.16); at first hyaline to bright brownish (P 15-1225), later brown (P 16-1422/17-1430) to dark brown (P 17-1422) (resembling *T. melanosporum* Vittad.); ornamented with straight or sometimes slightly curved, concolorous, dense, separate spines, 2–4(–4.5) µm high and 0.4–0.6 µm diam., 1–2.5 µm diam. at the base.

Odour of mature ascomata intensive and specific, pleasant, resembling a mixture of the aromas of *Tuber borchii* Vittad. and *Tuber brumale* Vittad.

Flavor not observed.

Additional specimens examined: **SERBIA, Bajina Bašta, Tara National Park**, Mitrovac, Dolak, 43.924810°N 19.462610°E, 1015 m asl, under *Fagus sylvatica* & *Abies alba*, 3 ascomata, 12 Oct. 2014, leg. M. Milenković & B. Ivančević (BEO 20606, 20607, 20608); Mitrovac, Tisovo brdo-Lokvica, 43.932980°N 19.448070°E, 1084 m asl, under *F. sylvatica*, *A. alba*, and *Picea abies*, 2 ascomata, 29 Sep. 2004, leg. M. Milenković (BEO 20601); 4 ascomata, 8 Oct. 2010, leg. M. Milenković (LJU GIS TUBPET/xx1010A, B, C, D; GenBank HG810883–810886); Mitrovac, Tisovo brdo, c. 43.927220°N 19.443649°E, 1075 m asl, under *F. sylvatica*, *A. alba* & *P. abies*, 1 ascoma, 29 Sep. 2004, leg. M. Milenković (herb. Marković); Mitrovac, Children's resort, 43.922180°N 19.421160°E, 1062 m, under *F. sylvatica* & *P. abies*, 3 ascomata, 5 Oct. 2006, leg. M. Milenković (herb. Marković); 1 ascoma, 11 Oct. 2014, leg. M. Milenković & B. Ivančević (BEO 20605); Šljivovica, Kovači, north slopes, 43.917050°N 19.491500°E, 1111 m, under *F. sylvatica* & *A. alba*, 2 ascomata, 9 Oct. 2010, leg. M. Milenković (herb. Marković); 2 ascomata, 11 Oct. 2014, leg. M. Milenković & B. Ivančević (BEO 20603, 20604); Šljivovica, Rustine, 43.912210°N 19.502850°E, 1029 m, under *F. sylvatica*, *A. alba* & *P. abies*, 2 ascomata, 18 Oct. 2010, leg. M. Milenković (herb. Marković); 1 ascoma, 11 Oct. 2014, leg. M. Milenković & B. Ivančević (BEO 20602).

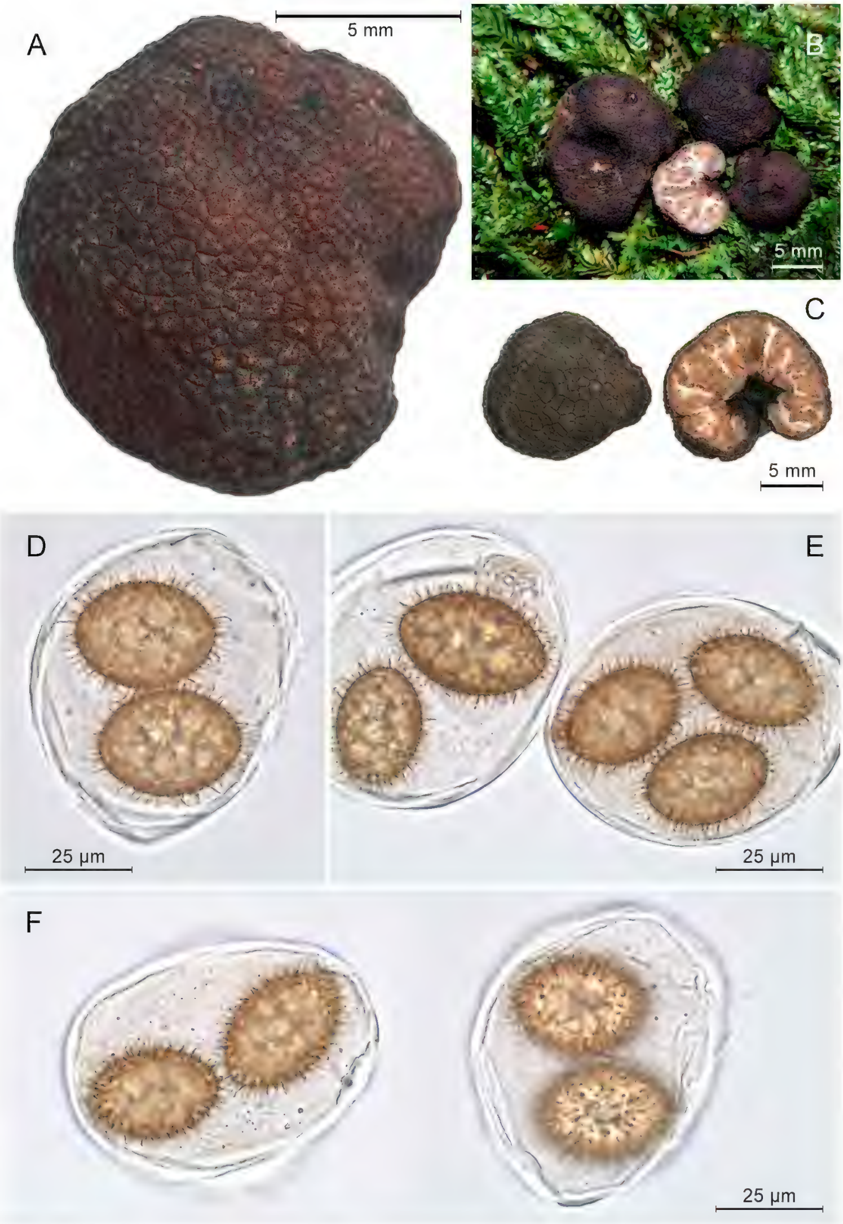

Plate 1. *Tuber petrophilum*: A. Fresh ascoma (BEO 20604). B, C. Fresh ascomata and cross section (B: BEO 20600: C: BEO 20608). D–F. Asci with spores from fresh ascomata (D, F: BEO 20608; E: BEO 20600).

HABITAT — All ascomata were found in humus-rich substrata accumulated as soil pockets in limestone rocks, 5–12 cm below the surface of the substratum and commonly 20–100 cm above the continuous forest soil terraces, formed on limestone mountain slopes of Mount Tara. The forest type is the continental montane forest dominated with *Fagus sylvatica* L., *Abies alba* Mill., and *Picea abies* (L.) H. Karst. forming an association *Piceo-Abietetum* Čolić 1965 (Tomić 2006, Tomić & Rakonjac 2011) at altitudes from 1000–1100 m asl.

PHENOLOGY — September to October.

DISTRIBUTION — Known only from the type locality and its environs in Serbia.

Discussion

Tuber P. Micheli ex F.H. Wigg. is among the best studied hypogeous genera in Europe (Hall et al. 2003). Until recently, about 32 different *Tuber* species had been described in Europe (Ceruti et al. 2003) and at least 180 species estimated worldwide (Bonito et al. 2010). *Tuber petrophilum*, unlike other hypogeous fungi, inhabits accumulated soil pockets in rocks, partly isolated from the surrounding continuous soil layer.

A combination of macro- and microscopic characters together with its unique ecology separate *Tuber petrophilum* from all other taxa in the *T. melanosporum* lineage (Riousset et al. 2001, Bonito et al. 2013), and its mature ascomata are considerably smaller than any other closely related species. Molecular data support its position in the *T. melanosporum* lineage and significantly support its separation from close relatives *T. brumale* aggr. and *T. pseudoexcavatum* Y. Wang et al. Spore size range and ornamentation size are key microscopic characters that distinguish it from species in the *T. melanosporum* lineage.

Tuber petrophilum forms a separate, well supported clade in the nrITS1-5.8S-ITS2-based phylogeny (PLATE 2). The closest ITS-based relatives are the European *Tuber brumale* aggr., *T. pseudoexcavatum* (Asia), *T. regimontanum* Guevara et al. (Central America), and *T. melanosporum* (Europe). We have observed a persistent presence of a c. 300-bp intron in the ITS1 region of *T. petrophilum* and *T. brumale* aggr. but not in other species in the *T. melanosporum* lineage. Excluding the intron, *T. petrophilum* shared 82–85% sequence similarity with other lineages within the *T. brumale* aggr. and ≤82% similarity with other species in the *T. melanosporum* lineage.

The ecology of *T. petrophilum* is strikingly distinct within the *T. melanosporum* lineage. While other species are found primarily in Mediterranean or Continental climates with both hot summer and moderate winter periods (Montecchi & Sarasini 2000, Riousset et al. 2001), *T. petrophilum* was discovered in a colder, montane climate at higher elevations and on different soil types. In

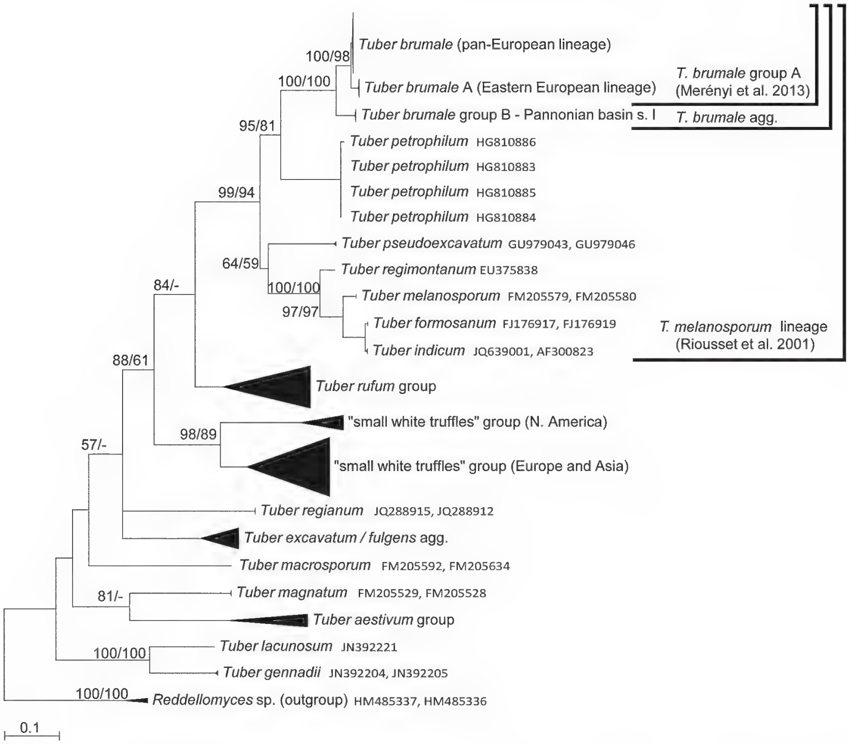
100/98
Tuber brumale (pan-European lineage)
100/100
Tuber brumale A (Eastern European lineage)
T. brumale group A (Merényi et al. 2013)
Tuber brumale group B - Pannonian basin s. l
T. brumale agg.
95/81
Tuber petrophilum HG810886
Tuber petrophilum HG810883
Tuber petrophilum HG810885
Tuber petrophilum HG810884
99/94
Tuber pseudoexcavatum GU979043, GU979046
64/59
Tuber regimontanum EU375838
84/-
100/100
Tuber melanosporum FM205579, FM205580
97/97
Tuber formosanum FJ176917, FJ176919
T. melanosporum lineage (Riousset et al. 2001)
Tuber indicum JQ639001, AF300823
88/61
Tuber rufum group
"small white truffles" group (N. America)
98/89
57/-
"small white truffles" group (Europe and Asia)
Tuber regianum JQ288915, JQ288912
Tuber excavatum / fulgens agg.
Tuber macrosporum FM205592, FM205634
81/-
Tuber magnatum FM205529, FM205528
Tuber aestivum group
100/100
Tuber lacunosum JN392221
Tuber gennadii JN392204, JN392205
100/100
Reddellomyces sp. (outgroup) HM485337, HM485336
0.1

PLATE 2. nr ITS1-5.8S-ITS2 phylogenetic reconstruction of the genus *Tuber* with a particular focus on the *T. melanosporum* lineage, with *T. petrophilum* as a neighbouring clade to *T. brumale* aggr. and *T. pseudoexcavatum*. The phylogenetic reconstruction is based on 140 sequences selected from well characterised collections deposited in the International Nucleotide Sequence Databases. Values for ML and MP branch supports are given for nodes with ≥50 bootstrap support.

particular, pH and humus contents deviate from requirements and ecological limits of other European species in the *T. melanosporum* lineage (Callot et al. 1999, Riousset et al. 2001). Site conditions for *T. petrophilum* are unique: its small size permits it to fruit well in small, humus-rich, and slightly acidic accumulations of soil in rock pockets that are physically separated from nearby soils. All specimens collected at different sites in the course of ten years were found in such pockets (cavities) or crevices in larger and smaller limestone rocks jutting from the soil, commonly 20–100 cm above the surrounding soil (PLATE 3). No ascomata were collected in the nearby continuous soil

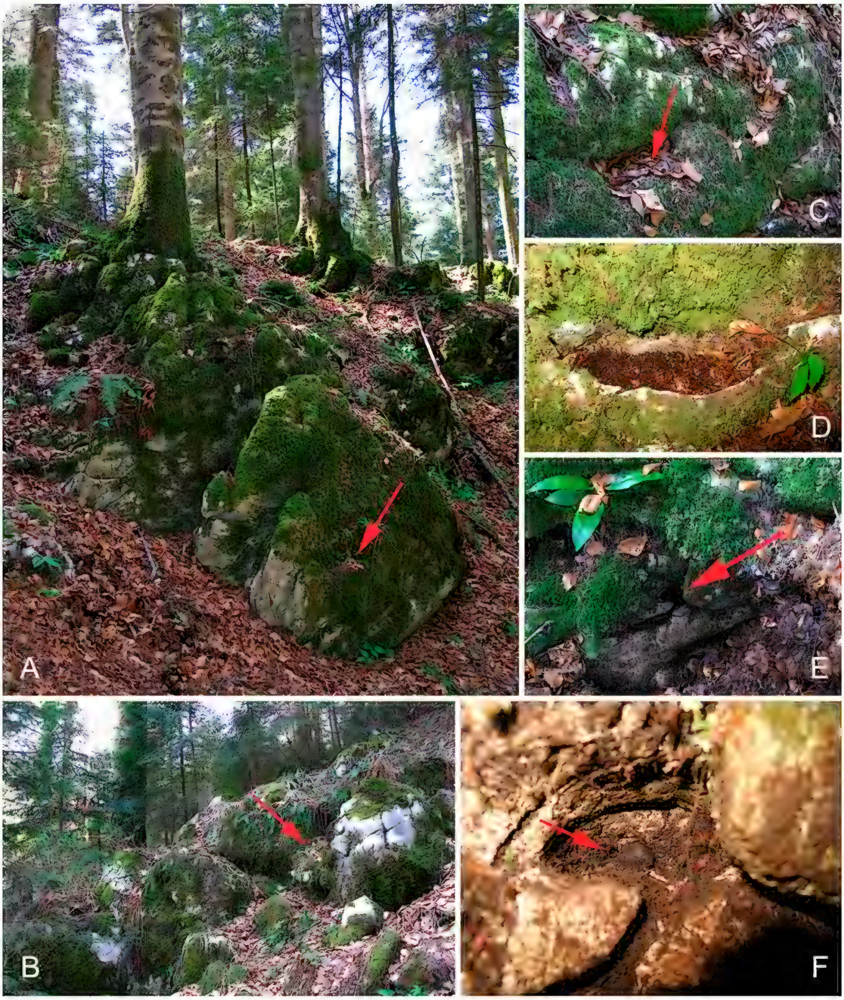

Plate 3. *Tuber petrophilum*: Typical microhabitat inside humus rich soil pocket in limestone rocks. A–C. Red arrows indicate the exact location where ascomata were unearthed. D. The substrate inside the hole is visible after removing the litter. E, F. The cavity containing ascomata.

layer, despite careful searching with the aid of a trained dog. In this unique microhabitat, humus mixed with eroded stone particles form a black, loose and well-aerated substratum. Chemical analysis of the *T. petrophilum* substratum confirmed high humus content (11.8–13.6%), with phosphorus measured at 5.15–11.70 mg/100 g and potassium at 24.35–34.06 mg/100 g. The pH of the substratum ranged from 6.3 to 7.0. This indicates that *T. petrophilum* is a highly demanding species with regard to the habitat requirements, which include presence of adequate rock cavities with a humus-rich substratum and simultaneously accessible to mycorrhizal roots of an appropriate tree species, which we assume to be *Fagus sylvatica*. Fructification appears limited to years with sufficient precipitation throughout the year, as mature ascomata were found in September and October (until snowfall) only in years without prolonged drought. The sporadic and irregular fruiting of only a small number of ascomata is probably caused by the combination of the karstic habitat and climatic conditions.

Tuber petrophilum has been found in only a few localities within a very small area, suggesting that it is a very rare species. Although *Fagus sylvatica* along with *Abies alba* stands are not rare in Serbia and throughout the Dinarides, here in combination with karst terrain, rocks, and soil a unique habitat was formed. Sites are near the road, in a National Park zone with the lowest regime of protection where forest exploitation is allowed, and felling was launched in the immediate vicinity of where the new species was found. On that basis, *T. petrophilum* is clearly threatened given its small known population and endangered habitat. Research on distribution, population trends, and ecological requirements of the species is needed, as well as protection measures and monitoring for the known population and sites.

The small size of the *T. petrophilum* ascomata and its relatively scarce fruiting is unlikely to establish the species as commercially or gastronomically significant, despite the high quality of its aroma.

Key to the species of *Tuber melanosporum* lineage

The *T. melanosporum* lineage differs from other lineages in the genus *Tuber* by black colour of the mature ascomata with spores bearing more or less pronounced warts or aculei.

1. Mature spores light brown to hazel brown, spines 3–6 µm long, thin;
gleba with few or infrequent branching veins. 2
Mature spores hazel brown to dark brown (when mature),
spines 1.5–8 µm long, variably thick; gleba with few or many thin veins. 4

2. Spores 33–55(–62) × 23–31 µm, Q = 1.3–2.0, spines 2–5 µm long, thin;
1–4 spores per ascus; gleba solid, dark brown;
described from Central America (Guevara et al. 2008) . . . . *T. regimontanum*
Spores generally smaller, 1–6 spores per ascus, Q not exceeding 1.8 3

3. Spores 25–31 × 16.5–18.5 µm, pan-European distribution,
ITS-supported clade (Merényi et al. 2014) . . . . *T. brumale* Group A
Spores 25–34 × 15.5–19.5 µm, Pannonian basin (Europe) distribution,
ITS-supported clade (Merényi et al. 2014) . . . . *T. brumale* Group B

4. Spores dark brown, opaque; ascomata prominent, roundish to irregular, lobed, never with basal depression or cavity. 5

   Spores brown to dark brown; ascomata with cavity or basal depression emerging from the basal point 6

5. Spores (20–)25–55 × 14–35 µm, spines 2–4(–6) µm long, thin, without wide base; 1–6 spores per ascus, Q = 1.4–2.1; gleba blackish brown with purple tinges, firm, with well-defined veins, reddening; aroma pleasant and characteristic, Mediterranean Europe (introduced worldwide). . . . . *T. melanosporum*

   Spores 15–35(–40) × 10–30(–35) µm, spines robust, curved, 3–5 µm long, with wide base and forming incomplete meshes of a reticulum; 1–6 spores per ascus; gleba blackish brown, compact, veins well defined, infrequent, thin; described from Asia. [Species an aggregate of several phylogenetic species (Chen et al. 2011)] . . . . *T. indicum* Cooke & Massee

6. Spores 12.5–25 × 10–18 µm, spines 3 µm long, truncate; gleba dark brown to black, with white to yellowish brown or purplish veins; described from *Fabaceae* and *Lauraceae* forests in southeast Asia (Huang et al. 2009) . . . . *T. formosanum* H.T. Hu & Y. Wang

   Spores >23 µm long, brown to dark brown; ascomata from native forests in Europe or (when from Asia) then ascomata deeply excavated; aroma intense, pleasant 7

7. Spores 23–42.5 × 13–26 µm, spines straight or slightly curved, 2–4(–4.5) µm long; (1–)–4(–6) spores per ascus; gleba dark brown with wide, 1–3× branched white veins that radiate from the base and end close to peridium; aroma pleasant resembling mixture of *T. borchii* and *T. brumale;* described from the eastern edge of Dinaric mountains (W. Serbia) . . . . *T. petrophilum*

   Spores (23–)24–28(–35) × 16–19(–22) µm, spines prominent, 5–8 µm long; 1–8 spores per ascus; gleba grey-brown with white sinuous veins; aroma intense; Asia, but introduced worldwide . . . .*T. pseudoexcavatum*

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